Biosynthesis of Conjugated Linoleic Acid in Humans

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ABSTRACT: This paper deals with the reanalysis of serum lipids from previous studies in which deuterated fatty acids were administered to a single person. Samples were reanalyzed to determine if the deuterated fatty acids were converted to deuterium-labeled conjugated linoleic acid (CLA, 9*c*,11*t*-18:2) or other CLA isomers. We found 11-*trans*-octadecenoate (fed as the triglyceride) was converted (Δ9 desaturase) to CLA, at a CLA enrichment of *ca.* 30%. The 11-*cis*-octadecenoate isomer was also converted to 9*c*,11*c*-18:2, but at <10% the concentration of the 11*t*-18:1 isomer. No evidence (within our limits of detection) for conversion of 10-*cis*- or 10-*trans*-octadecenoate to the 10,12-CLA isomers (Δ12 desaturase) was found. No evidence for the conversion of 9-*cis*,12-*cis*-octadecadienoate to CLA (*via* isomerase enzyme) was found. Although these data come from four single human subject studies, data from some 30 similar human studies have convinced us that the existence of a metabolic pathway in one subject may be extrapolated to the normal adult population.

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Conjugated linoleic acid (CLA; 9-*cis*,11-*trans*-octadecadienoic acid; 9*c*,11*t*-18:2) has been associated with the reduction of chemically induced cancers in mice and rats and the suppression of atherosclerosis in rats (1–3). Whereas commercially available samples of CLA usually contain a mixture of conjugated fatty acid (FA) isomers (4,5), the 9-*cis*,11-*trans*-octadecadienoic acid isomer is considered to be the active constituent (6). Rats fed 11*t*-18:1 were shown to have increased levels of CLA in their tissues (7), and the presence of a similar metabolic pathway (11*t*-18:1 → 9*c*,11*t*-18:2 *via* Δ9 desaturase enzyme) has been suggested to occur in humans (8–11). Furthermore, Chin *et al.* (12) demonstrated that both the 9*c*,11*t*- and 10*t*,12*c*-18:2 FA isomers are produced in conventional, but not germ-free, rats fed linoleic acid and described the conversion of 9*c*,12*c*-18:2 to 9*c*,11*t*-18:2. (See Reference 13 for an extensive review of CLA biosynthesis.)

This study was undertaken to determine if, as postulated, detectable amounts [defined as present at >2 ng fatty acid methyl esters (FAME)/mL plasma] of the previously mentioned FA are actually converted to CLA or CLA isomers (i.e., 10*t*,12*c*-18:2) in humans. Samples from human metabolism studies in which deuterium-labeled fats were fed as triglycerides (TG) were reanalyzed for evidence of the following conversions: (i) 11*c*-18:1 to 9*c*,11*c*-18:2, (ii) 11*t*-18:1 to 9*c*,11*t*-18:2, (iii) 10*c*-18:1 to 10*c*,12*c*-18:2, (iv) 10*t*-18:1 to 10*t*,12*c*-18:2, and (v) 9*c*,12*c*-18:2 to 9*c*,11*t*-18:2. [The deuterium label is nonradioactive; thus deuterium-labeled fats can be safely fed to human volunteers (14–16).] Since conjugated 18:2 FAME isomers tend to elute by gas chromatography (GC) later than their methylene-interrupted analogs, the time windows set for selective ion monitoring in the original GC/mass spectrometry (MS) studies (Table 1) were not set to detect the CLA ions. By reanalysis of relevant samples, we were able to demonstrate the existence or absence of these biosynthetic pathways in humans. It must again be emphasized that each study is limited to a single human subject.

EXPERIMENTAL PROCEDURES

Materials. All reagents used were analytical grade or better.

Procedures/equipment. (i) Protocol of original studies. Complete details may be found in References 17–21. In these studies (designated I, II, III, and IV in Table 1), subjects were fed milkshakes containing a mixture of 8–10 g (studies I, II, III) or 2.5 g (study IV) each of 3–5 deuterium-labeled FA. The labeled fats were fed as homogeneous TG. (See Table 1 for spe-

TABLE 1
Deuterium-Labeled Fatty Acid Contents of Triglyceride Mixtures Fed

Study	Date	No. subjects	Fed	Reference(s)
I	1978	1	9*c*-18:1-9,10-d_2 11*t*-18:1-15,15,16,16-d_4 11*c*-18:1-14,14,15,15,17,18-d_6	17
II	1979	1	9*c*-18:1-14,14,15,15,17,18-d_6 10*t*-18:1-9,10-d_2 10*c*-18:1-15,15,16,16-d_4	18
III	1980	1	9*c*-18:1-14,14,15,15,17,18-d_6 10*t*-18:1-15,15,16,16-d_4 10*c*-18:1-9,10-d_2	18
IV	1988	1	16:0-9,10-d_2 18:0-9,10-d_2 9*c*-18:1-14,14,15,15,17,18-d_6 9*c*,12*c*-18:2-15,15,16,16-d_4 9*c*,12*c*,15*c*-18:3-9,10-d_2	19–21

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Abbreviations: CLA, conjugated linoleic acid; 9-*cis*,11-*trans*-octadecadienoic acid; 9c,11t-18:2, EE, ethyl ether; FA, fatty acid(s), FAME, fatty acid methyl ester(s), GC, gas chromatography, GC/MS, gas chromatography/mass spectrometry, PE, petroleum ether; PL phospholipid(s); TG triglyceride(s); TLC, thin-layer chromatography.

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cific fats fed.) Blood samples were collected by venipuncture at specified intervals (i.e., 0, 2, 4, 8, 12, 15, 24, 48 h), and the red blood cells were removed by centrifugation. One portion of the blood plasma was extracted with $CHCl_3$/methanol to obtain total plasma lipids, from which the neutral and phospholipid (PL) classes were separated by preparative thin-layer chromatography (TLC). An internal standard (17:0) was added to each fraction. The lipids in each fraction were converted to FAME with HCl/methanol (22), and each fraction was analyzed by GC to obtain total lipid composition and by GC/MS [chemical ionization conditions with isobutane as the reagent gas and selective ion monitoring (23,24)] to determine the deuterium-labeled FA composition. The blood lipid FAME samples were stored in isooctane under nitrogen gas in 1-dram vials (aluminum foil-lined caps) at 0°C (in the dark).

(ii) Sample selection. To maximize GC/MS sensitivity, samples were taken from studies in which the fats of interest fed were labeled with four or more deuterium atoms. The carbon-13 isotope contribution to the MS data for FA labeled with two deuterium atoms must be calculated, resulting in some loss of sensitivity. Given the low (0.1–0.3%) levels of CLA isomers present, the authors felt the highest sensitivity/accuracy would be obtained by analyzing for fats labeled with four or more deuterium atoms. A representative grouping of 4–6 blood lipid samples (plasma TG/PL or chylomicron TG/PL) from each of four different human metabolism studies were retrieved. The date of the study, the FA fed and pertinent publications resulting from these studies are listed in Table 1.

(iii) Sample preparation. Each sample was eluted through a Sep- Pak cartridge (Waters, Inc., Milford, MA) with 10 mL 10% ethyl ether (EE) in petroleum ether (PE; vol/vol) as solvent. The eluant was concentrated to 0.5 mL under a stream of inert gas and analyzed by TLC (Silica gel 60A, Whatman, Inc., Clifton, NJ), using 15% EE/85% PE as solvent and with I_2 visualization. The remaining solvents were removed by a stream of inert gas, and the residue was transferred to a sample vial with a minimal volume of isooctane for analysis by GC.

(iv) Blood lipid fraction analysis by GC. Samples (as FAME) were initially analyzed on a Varian 3400 GC (Varian Instruments, Palo Alto, CA) to obtain a total lipid profile. The GC was equipped with a 100 m × 0.32 mm SP2380 (Supelco, Inc., Bellefonte, PA) capillary column and flame-ionization detector (FID). Helium was utilized as carrier gas. Unknown peaks were identified by comparison with standard FAME mixtures of known composition. The extent of sample oxidation was estimated by comparing saturated and polyunsaturated FA compositions from our GC results with the original published data.

(v) GC/MS analysis of blood lipid fractions. GC/MS was utilized to determine the presence (or absence) of deuterium-labeled CLA and other deuterium-labeled metabolites. Analyses were made on a Hewlett-Packard model 5890/5988a GC/MS (quadrapole; positive chemical ionization mode; isobutane as ionizing gas; Palo Alto, CA) equipped with a 30 m × 0.25 mm Omegawax 10 fused-silica capillary column (Supelco, Inc.), Data collection and processing have been described previously (23). The isomerization of conjugated FA during their conversion to FAME by acidic catalysts such as HCl or BF_3 in methanol has been well documented (5,25). Since 5% HCl in methanol (at 50°C) had initially been used to convert the blood lipids to FAME (22), the 9*c*,11*t*- and 9*t*,11*t*-18:2-d_4 and -d_0 peak areas were combined to calculate total concentrations of labeled and unlabeled CLA, respectively. If deuterium-labeled CLA was detected, additional samples from that study were retrieved and prepared for analysis by GC and GC/MS as described previously.

RESULTS

Only in study I (in which 11*c*-18:1-d_6 and 11*t*-18:1-d_4 were fed) were deuterium-labeled CLA isomers detected in measurable (>2 ng FAME/mL plasma) quantities. Four samples (0-, 4-, 8-, and 15-h) of blood plasma TG (as FAME) were retrieved and reanalyzed by GC and GC/MS to provide the data presented in this study. A portion of a gas chromatogram (100 m SP2380 capillary column) containing the CLA isomers is shown in Figure 1. Peaks have been identified by coinjection with known standards or marked as "unknown."

The percentage of 9*t*,11*t*-18:2 formed during conversion (HCl/methanol) of the blood TG fractions to FAME varied significantly (from 10 to *ca.* 50% of total CLA concentration) from sample to sample. This amount of isomerization is typical when HCl/methanol or BF_3/methanol and 50°C are used to convert CLA-containing TG, PL, or free FA to FAME. The two peak areas were combined to calculate total % CLA. A representative GC/MS chromatogram, with the peaks of interest labeled, of the CLA region for the 4-h plasma TG sample is presented in Figure 2. As we demonstrate in Figure 2, the presence of deuterium-labeled CLA and CLA isomers may readily be detected by our methodology. The 9*c*,11*c*-18:2 isomer is also present, but at <0.01%. We also characterized the separation

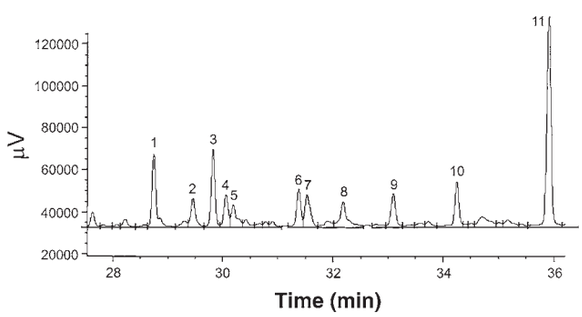

FIG. 1. Portion of gas chromatography trace (100 m SP2380 capillary column; Supelco Inc., Bellefonte, PA) with conjugated linoleic acid (CLA) and other minor (<1% each by weight total lipids) fatty acid methyl esters marked. (See Experimental Procedures section for details.) Designations: peak no. 1 = 6*c*,9*c*,12*c*-18:3; peak no. 2 = 11*c*-20:1, peak no. 3 = 9*c*,12*c*,15*c*-18:3; peak no. 4 = 9*c*,11*t*-18:2; peak no. 5 = 21:0; peak no. 6 = 9*t*,11*t*-18:2; peak no. 7 = unknown (8*c*,11*c*-20:2?); peak no. 8 = 11*c*,14*c*-20:2; peak no. 9 = 22:0; peak no. 10 = 8*c*,11*c*,14*c*-20:3; peak no. 11 = 5*c*,8*c*,11*c*,14*c*-20:4.

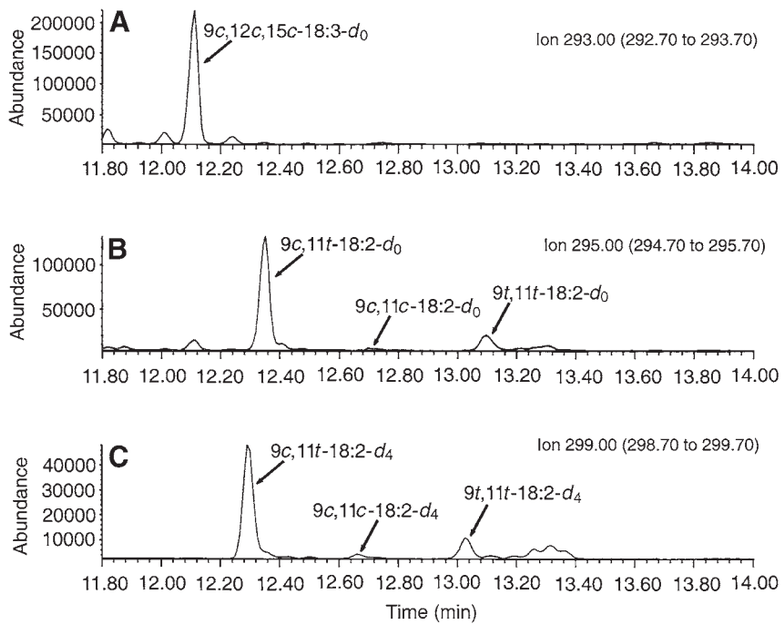

FIG. 2. Portion of gas chromatography/mass spectrometry chromatogram illustrating selective ion monitoring at molecular weights 292, 294, and 298 (molecular ions of 293, 295, and 299), corresponding to 18:3n-3-d_0, 9*c*,11*t*-18:2-d_0, and 9*c*,11*t*-18:2-d_4, respectively. (See Experimental Procedures section for details.)

capabilities of the Omegawax GC column by injecting a mixture of 9,11- and 10,12-18:2 FAME isomers. Under the GC conditions employed (see the Experimental Procedures section), we found the Omegawax column capable of achieving baseline separation of 9*c*,11*t*-18:2 and 10*t*,12*c*-18:2, the 9*c*-isomer eluting before the 10*t*-isomer. The 9*c*,11*c*-18:2 and 10*c*,12*c*-18:2 were only slightly resolved, with the 9*c*-isomer eluting first. The 9*t*,11*t*-18:2 and 10*t*,12*t*-18:2 isomer pair was not resolved.

The incorporation of 11*t*-18:1-d_4 and its conversion to CLA-d_4 and 9*t*-16:1-d_4 (formed by β-oxidation of 11*t*-18:1-d_4) are plotted (as enrichment data) in Figure 3. A small amount of 9*c*,11*c*-18:2-d_6 (from 11*c*-18:1-d_6 *via* Δ9 desaturase) was also detected at *ca.* 10% of the CLA-d_4 concentration. The total weight percentages of the isomers (labeled and unlabeled) plotted in Figure 3 are presented in Table 2.

DISCUSSION

After 10–20 yr in storage, some oxidation of the lipid samples was expected. Although some loss (2–5%) of polyunsaturated FA was noted, the overall lipid compositions were found to compare favorably with the original lipid data obtained by packed column GC (data not shown). A more limited selection of samples from studies II, III and IV was also analyzed for extent of oxidation. Since the oxidative stability of CLA has been reported to lie between arachidonic and docosahexaenoic acids (26), the 20:4 FA concentration was chosen as another marker for oxidation. The low concentration of this FA (0.8–2.2%), however, limited this approach. The formation of methoxy artifacts during the FAME preparation or the presence of plasticizers was also studied. By use of internal standards, we found methoxy artifacts to be formed at a total concentration of <0.5% during our HCl/methanol procedure. Plasticizers were also detected during our GC analyses, but at a total concentration of <0.01%. Any possible interference(s) due to methoxy artifacts or plasticizers were eliminated by use of selective ion monitoring (Fig. 2).

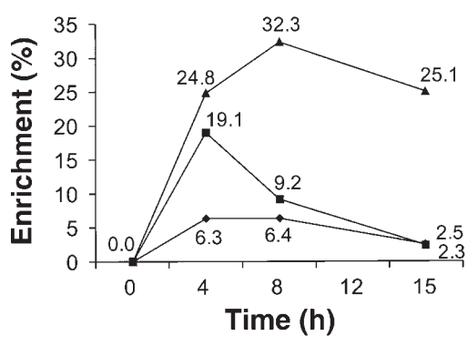

FIG. 3. Uptake and disappearance in plasma triglycerides of 11*t*-18:1-d_4 (■) related to formation and disappearance of 9*c*,11*t*-18:2-d_4 (▲) and 9*t*-16:1-d_4 (◆). Data plotted as percent isotopic enrichment within each individual fatty acid methyl ester.

TABLE 2
Study I: Compositions (percentage by weight) for Fatty Acids Plotted in Figure 3

	Hour			
Fatty acid[a]	0	4	8	15
9*t*-16:1	2.0	1.5	1.5	1.5
11*t*-18:1	1.4	11.4	4.5	2.5
CLA	0.07	0.19	0.32	0.14

[a]Total (deuterium-labeled and unlabeled) percentage of fatty acids. CLA, conjugated linoleic acid.

The only readily identifiable metabolic pathway for the biosynthesis of CLA in humans utilizes the Δ11-18:1 FA as precursors and, presumably, the Δ9 desaturase enzyme (10). As we demonstrated in study I (17), both the 9*c*-18:1 and the Δ11-18:1 isomers are equally well-absorbed in humans. The total percentage of CLA in blood plasma lipids 8 h after ingestion of more than 8 g of 11*t*-18:1 (fed as a homogeneous TG) was found to be only 0.3% by weight of total blood lipids. This number (0.3%) falls within the percentage composition ranges listed for CLA in blood lipids by Ackman (27) and Jiang *et al.* (28). At 33% enrichment, the CLA-d_4 was present at a maximum of only 0.1% (by weight) of total lipids. The presence of 9*c*,11*c*-18:2-14,14,15,15,17,18-d_6 at *ca.* 0.01% by weight suggests the existence of a similar, but less utilized, biosynthetic pathway for conversion of the 11*c*-18:1 isomer. No CLA-d_4 metabolites (6*c*,9*c*,11*t*-18:3-d_4 or 5*c*,8*c*,11*c*,13*t*-20:4-d_4) were detected. [In the original study (17), no metabolites of the Δ11-18:1 isomers were found (0.001% listed then as limit of detectability)].

It should again be stressed that a single human male was used in each of the feeding studies listed in Table I; the data thus cannot be statistically evaluated as representative of the normal population. The amounts of CLA formed from the 11*t*-18:1 isomer are expected to vary between individuals, but data from some 30 human studies have convinced us (17) that the existence of a metabolic pathway in one subject may be safely extrapolated to the normal adult population. Our results also agree with previous findings which show 9*c*,11*t*-18:1 to be "practically the only one of the CLA isomers found in humans" (10). Other postulated pathways for the biosynthesis of CLA isomers in humans (10*t*,12*c*-18:2 from 10*t*-18:1 or 10*c*,12*c*-18:2 from 10*c*-18:1, both by Δ12 desaturase) are still feasible but, if these isomers were formed, they were formed below the detection limits (again defined as <2 ng FAME/mL plasma) of our methodologies.

The conversion of 9*c*,12*c*-18:2 to CLA *via* an isomerase enzyme generated by bacteria in the human intestinal tract [as noted by Chin *et al.* in mice (12)] also remains a possibility, but any deuterium-labeled CLA formed in the human colon would be expected to be as poorly absorbed in the lower intestine of animals as are methylene-interrupted polyunsaturated FA (8). Previous studies in humans (19,29) have shown ingested 9*c*,12*c*-18:2 (as TG) to be >96% absorbed. Only a very small percentage of the labeled 9*c*,12*c*-18:2 fed would thus be available to bacteria in the intestine. Any metabolites formed *via* isomerization of 9*c*,12*c*-18:2 and absorbed in the lower intestine would again be present at levels below our limits of detection.

All other sources/pathways to explain the presence of deuterium-labeled CLA isomers (impurities in the fed materials, oxidation, etc.) in study I samples have been examined and eliminated. Utilizing isotopically labeled FA, we thus demonstrate the existence, in human(s), of a metabolic pathway for the biosynthesis of CLA. Since some oxidation may have occurred during storage, the concentrations of CLA presented in this manuscript should be considered at the low end of possible CLA levels in human plasma. The relatively large (>8 g) quantity of 11*t*-18:1 ingested also resulted in a relatively small increase in plasma CLA concentration (from 0.1 to 0.3% total lipids). Whether the amount of CLA formed from increased levels of 11*t*-18:1 in the diet will result in "positive health benefits" and a situation where "*trans* FA in dairy products represent a complete opposite situation to the possible adverse health effects of *trans* FA isomers from partially hydrogenated plant oils" (30) remains to be seen.

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